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13. ABSTRACT (Maximum 200 Words) Since collagenases (-1 and -3) degrade the extracellular bone matrix (ECM) components, collagenase-driven ECM proteolysis may facilitate cancer growth and progression. To test this hypothesis, we made a transgene construct containing collagenase-1 cDNA under the control of the bone specific osteocalcin promoter. The application of a transgenic mouse model will contribute greatly to the understanding of the pathogenesis of bone metastasis. Transforming growth factor (TGF)- β 1 is a crucial molecule in metastatic breast cancer. It can potentially disrupt the normal balance between osteoclast- and osteoblast-derived matrix metalloproteinase (MMP) activity within bone by inducing the expression of MMPs and their inhibitors from bone-metastasizing cancer cells. TGF- β 1 stimulates collagenase-3 expression in human breast cancer cells (metastatic in nature). An intensive drug discovery program led to many clinical trials of MMP inhibitors for cancer therapy. However, these trials have largely been disappointing. A greater Understanding of the regulatory mechanisms that control MMP transcription, activation and inhibition will provide several new avenues for therapeutic intervention. Here, we dissected the signaling and molecular mechanisms responsible for TGF- β 1 stimulated collagenase-3 expression in human breast cancer cells.				
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Collagenases in Breast Cancer Cell-induced Metastatic Tumor Growth and Progression:

Introduction:

Matrix metalloproteinases (MMPs) appear to play an important role in the multiple steps of breast cancer development and metastasis (1-4). Since collagenases-1 and -3 (MMP-1 and -13) are characterized by their potent ability to degrade the extracellular bone matrix (ECM), it is likely that collagenase-driven ECM proteolysis supports cancer cell expansion both biochemically by exposing mitogenic factors and physically by providing space for the proliferating cells. It is also possible that growth factors and cytokines released from the ECM could act in a feed-forward manner by inducing cancer cells to secrete more collagenases.

Bone is one of the major sites for formation of breast cancer metastases. The molecular mechanisms responsible for osteolytic metastases are complex and involve bi-directional interactions between tumor cells and bone. TGF- β 1 (transforming growth factor-- β 1) is a crucial molecule in metastatic breast cancer. It is released as a result of bone resorption and may alter the nature of tumor cells, resulting in more aggressive local bone resorption and osteolysis. TGF- β 1 can potentially disrupt the normal balance between osteoclast- and osteoblast-derived MMP activity within bone by inducing the expression of MMPs and their inhibitors from bone-metastasizing cancer cells.

Body:

In order to study the role of collagenases on cancer growth and progression, we proposed to utilize a transgenic mouse model to overexpress collagenase under the control of the bone specific osteocalcin promoter. The osteocalcin promoter has been shown to confer differentiated osteoblast- and post-specific expression to a reporter gene *in vivo*. To generate transgenic mice overexpressing collagenase, we first initiated our work to clone the human collagenase-1 cDNA (1.65 kb) and express it *in vitro*. We used pcDNA3.1 Directional TOPO Expression construct (Invitrogen) for this purpose. The pcDNA3.1 contains the following elements: human cytomegalovirus (CMV) immediate-early promoter/enhancer that permits efficient, high-level expression of recombinant protein and V5 epitope that allows detection of recombinant protein with anti-V5 antibody. The collagenase-1 cDNA followed by a V5-epitope tag was cloned downstream into the CMV promoter sequence. The construct pCMV-MMP-1-V5 was transfected into COS-7 cells using the Lipofectamine 2000 reagent (Invitrogen) according to the guidelines provided by the company. Cells were lysed and subjected to Western blot analysis. The results indicate that collagenase-1-V5 is efficiently expressed under the control of CMV promoter.

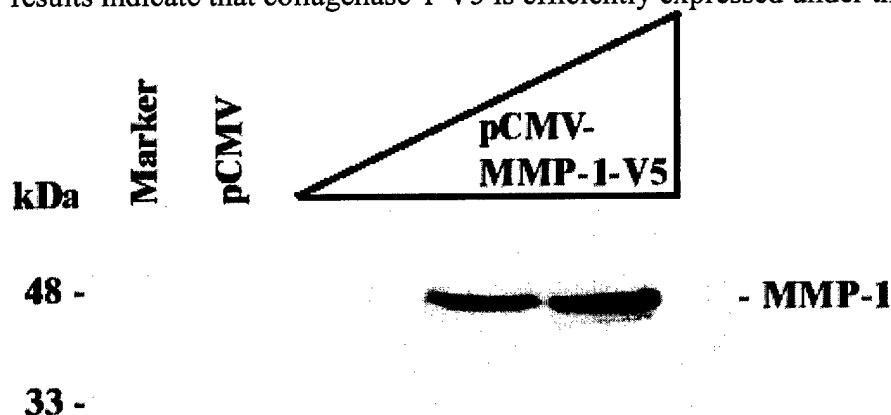


Figure 1. Expression of MMP-1 (Collagenase-1). The construct (pCMV-MMP-1-V5) at different concentrations was transfected into COS-7 cells using the Lipofectamine 2000 reagent for 24 h. The cells were then lysed. The lysates were subjected to 12% SDS polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes and the immunoblot was probed with anti-V5 monoclonal antibody.

In order to get bone specific expression of collagenase-1, we utilized the rat osteocalcin promoter (OC; ~1.0 kb). It was kindly provided by Dr. Gary Stein, University of Massachusetts Medical School, Worcester, MA. The pOC-collagenase-1-V5 was constructed by replacing the CMV promoter sequence from pCMV-MMP-1-V5 with the rat osteocalcin promoter sequence. The ability of osteocalcin promoter to drive collagenase-1-V5 expression was carried out by transient transfection assays and Western blot analysis. The 2.6 kbp DNA fragment containing the osteocalcin promoter and collagenase-1-V5 cDNA sequence was excised from pOC-collagenase-1-V5 with appropriate enzymes. This purified transgene is ready to use for generation of transgenic mice.

Figure 2. Cardiac injection and tumor analysis. My collaborator (Dr. Susan Rittling, Rutgers University, NJ) generated a series of metastatic murine mammary epithelial cell lines using normal mice rather than using nude mice. Mammary tumors were induced in strain 129 female mice by treatment with the carcinogen DMBA and their growth was accelerated by implanted progesterone (MPA) pellets.



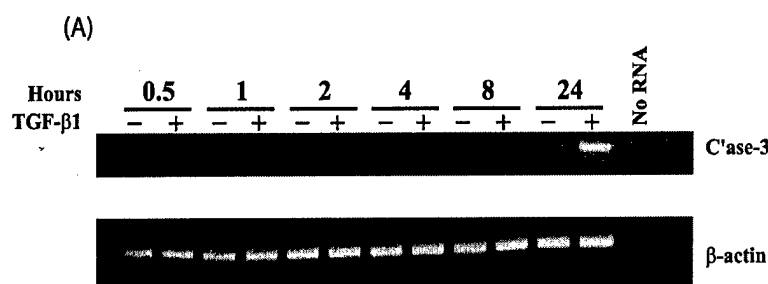
An epithelial cell line was isolated from one of these tumors: however, this cell line (1029) was not tumorigenic in mice even in the presence of MPA. Introduction of the oncogene polyoma middle T into these cells (1029 GP+E) was insufficient to allow hormone-independent tumor growth, although in the presence of hormone (MPA) rare tumors were observed. Thus, a second oncogene, v-Ha-ras was introduced. Cells expressing both ras and polyoma middle T were able to form tumors efficiently in the mammary fat pad, and even formed spontaneous metastases in the lungs and sometimes liver after mammary tumor growth (1029 GP+E r3). Two additional cell lines were

derived from the 1029 GP+E r3 cells – one from a tumor and one from a lung metastasis arising from injected cells: these cells were called r3T and r3L, respectively. These two cell lines have similar metastatic properties. To get expertise in the techniques of cardiac injection of cancer cells into the mice and tumor analysis, we utilized those cancer cells with normal mice. The r3T or r3L cells (5×10^5) were injected into the left ventricle (L.V.) of the heart, and the mice were sacrificed three weeks later. Arrows indicate positions of extensive bone loss visible in these x-ray images. The bone indicated by an asterisk was actually broken completely as a result of tumor growth.

Collagenase-3 (MMP-13) is overexpressed in a variety of malignant tumors. In the present study we show that TGF- β 1 stimulates collagenase-3 expression in the human breast cancer cell line MDA-MB231. Since collagenase-3 is characterized by its ability to degrade the ECM and is stimulated by TGF- β 1 in MDA-MB231 cells, collagenase-3 driven ECM proteolysis may support cancer cell growth both biochemically by exposing mitogenic factors and physically by providing space for the proliferating cells. To delineate the molecular mechanisms responsible for this stimulation of collagenase-3 by TGF- β in these cells, a functional analysis of the collagenase-3 promoter was carried out.

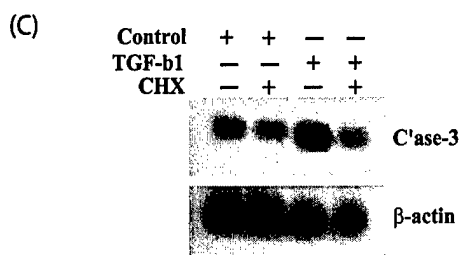
TGF- β 1 stimulation of collagenase-3 expression in MDA-MB231 cells requires *de novo* protein synthesis - To study the effect of TGF- β 1 on expression of collagenase-3 in human breast cancer, MDA-MB231 cells were treated with TGF- β 1 either at different concentrations for 24 h or for different time periods with TGF- β 1 (10 ng/ml). Total cellular RNAs were purified and analyzed by semi-quantitative RT-PCR. TGF- β 1 maximally stimulated collagenase-3 RNA expression at 10 ng/ml concentration in MDA-MB231 cells (data not shown) and requires at 24 h (Fig. 3A). The identity of

collagenase-3 (392 bp) and β -actin (661 bp) PCR products were confirmed by transferring PCR products to filters and hybridizing to labeled human collagenase-3 and β -actin cDNA probes, respectively.



(B)

Treatment	Collagenase-3 secretion (ng/ml)
Control	1.29 ± 0.069
TGF- β 1	4.22 ± 0.103^a



TGF- β 1 (10 ng/ml) for 24 h in the presence or absence of cycloheximide (30 μ g/ml, added 1 h before TGF- β 1 treatment). Total RNA was subjected to Northern blot analysis and probed with labeled human collagenase-3 or β -actin cDNA.

An ELISA was then performed using an antibody to human collagenase-3 confirming increased secretion of collagenase-3 into the medium from TGF- β 1-treated MDA-MB231 cells (Fig. 3B). To determine if the TGF- β -mediated increase in collagenase-3 mRNA is a primary response, we used the protein synthesis inhibitor cycloheximide. As shown in Fig. 3C, cycloheximide inhibited TGF- β 1 stimulation of collagenase-3 mRNA, indicating that *de novo* protein synthesis is required for this response.

The RD and AP-1 sites are necessary for TGF- β 1-stimulated collagenase-3 promoter activity - The collagenase-3 promoter region includes consensus binding sites for several DNA-binding proteins, C/EBP, SBE (smad binding element), RD (runt domain binding sequence), p53, PEA-3, AP-2, and AP-1 (activator protein-1) (5). There are four consensus sites, namely a RD site, a p53 site, a PEA site, and an AP-1 site, which are highly conserved both in sequence and location in both the human and rat collagenase-3 promoters. The RD binding site is identical to a Cbfa/Runx binding site and the proteins binding to this site are Cbfa/Runx transcription factors (6-8). Previously, we deleted regions of the rat collagenase-3 promoter from the 5'-end and placed the resulting promoter sequences 5' of the CAT (chloramphenicol acetyltransferase) gene. We also showed that the AP-1 site is mainly involved in basal expression; whereas the RD site is necessary for PTH-induced collagenase-3 promoter activity in the rat osteoblastic cell line, UMR 106-01 (5). Both the RD and AP-1 sites are contained within the 148 base pairs upstream of the transcriptional start site. The collagenase-3 promoter also contains another RD site (proximal) overlapping with the AP-1 site and its sequences (ACCAC) are similar to the Cbfa/Runx consensus site, ACC(A/G)CA (9). Even though the AP-1 site is conserved among the human and rat

Figure 3. TGF- β stimulation of collagenase-3 expression in MDA-MB231 cells requires *de novo* protein synthesis. A, MDA-MB231 cells were serum starved for 24 h and then treated with TGF- β 1 (10 ng/ml) at different time periods as indicated. Sense and antisense oligos for human collagenase-3 (C'ase-3) and β -actin were used for RT-PCR. B, MDA-MB231 cells were serum starved for 24 h and then treated with control medium or medium containing TGF- β 1 (10 ng/ml) for 24 h. The collagenase-3 levels in the media were measured using an ELISA kit (Amersham Pharmacia). Data represent mean \pm S. E. of three replicate plates. The statistical analysis was performed using Student's *t*-test and Prism 3.0. ^aSignificant difference compared with control ($P < 0.001$). C, MDA-MB231 cells were serum starved for 24 h and then treated with control medium or medium containing

collagenase-3 genes, the proximal RD site overlapping with the AP-1 site is located in the opposite orientation and on the opposite strand (Fig. 4A). Since it has been reported that the RD site acts as enhancer (10), the proximal RD site could also be a functional element in mediating the TGF- β -response.

In order to analyze the specific response elements involved in TGF- β 1-stimulated collagenase-3 promoter activity in MDA-MB231 cells, the promoter constructs having mutations at either the distal RD or the proximal RD or the AP-1 sites were used and the effect on CAT activity was assessed in these cells. Mutation of either the distal RD or the proximal RD or the AP-1 sites reduced the basal activity; whereas mutation at any one of those sites was enough to cause a significant loss of TGF- β 1-response for collagenase-3 promoter activity in the breast cancer cells (Fig. 4B). Hence, these results suggest that the distal RD and the proximal RD/AP-1 sites are necessary for full TGF- β 1-stimulated collagenase-3 promoter activity.

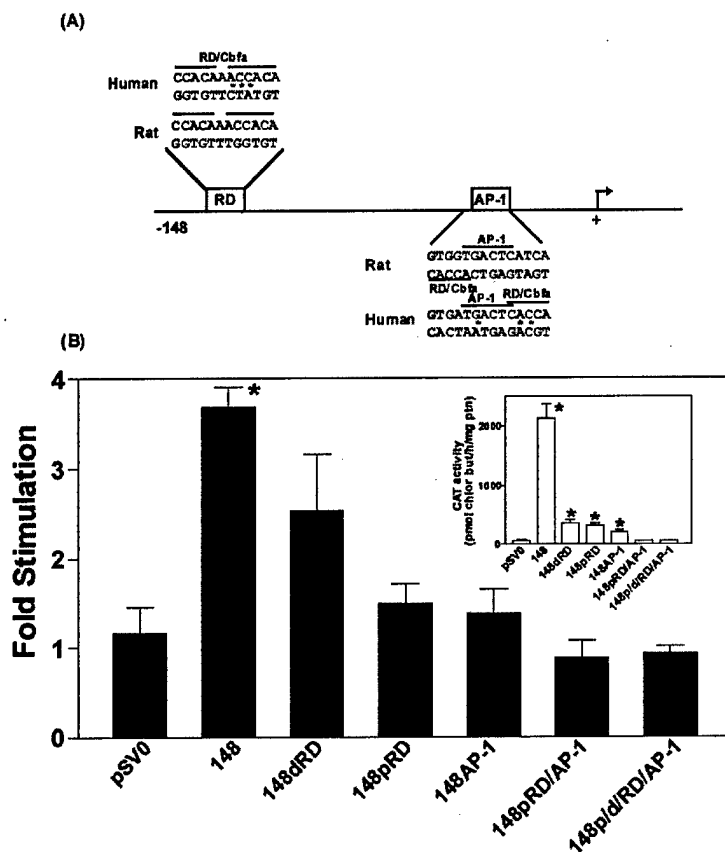


Figure 4. The RD and AP-1 sites are necessary for TGF- β 1-stimulated collagenase-3 promoter activity. A, Comparison of the nucleotide sequence of the distal RD and the proximal RD/AP-1 sites of the -148 rat and human collagenase-3 promoters (+ indicates the transcription initiation site). The asterisks indicate mutated nucleotides within human collagenase-3 distal RD, AP-1, and proximal RD sites. B, The wild type and mutant rat collagenase-3 promoter constructs were transiently transfected into MDA-MB231 cells, treated with control or TGF- β 1-containing media for 24 h, and then assayed for CAT activity. The TGF- β 1-response is shown as -fold stimulation over control. The inset graph shows the basal activity of collagenase-3 promoter constructs. Data represent mean \pm S.E. of three experiments.

*Significant difference compared with control (promoterless) vector, pSV0 ($P < 0.05$). (dRD-distal RD site; pRD-proximal RD site; AP-1-activator protein-1 site)

Dominant negative inhibitors identify the functional requirement of both the RD and AP-1 sites for TGF- β 1-stimulated collagenase-3 promoter activity - To determine the functional role of the RD sites and its transcription factor family, Cbfa, for TGF- β 1-stimulated collagenase-3 promoter activity in MDA-MB231 cells, we used AML/ETO, a chimeric protein caused by chromosomal translocation t(8;21) that lacks a transactivation domain at the carboxyterminal portion of AML1 and acts as a repressor of Cbfa proteins (5). Since mutations either at the distal or the proximal RD sites in the collagenase-3 promoter constructs inhibit the TGF- β 1-response (Fig. 4B), the wild type collagenase-3

promoter construct (-148) was used in these studies. The collagenase-3 promoter construct was transiently co-transfected into MDA-MB231 cells with an AML/ETO expression plasmid (Fig. 5). The result demonstrates that the basal and the TGF- β 1-response were greatly reduced by overexpression of AML/ETO, suggesting that both the RD site and Cbfa are required for collagenase-3 promoter activity in MDA-MB231 cells. Previously, we showed that there is a high level of expression of fra-1 in MDA-MB231 cells (11). Like c-Fos, Fra-1 also heterodimerizes with Jun family members. Hence, to identify the functional role of the AP-1 site and its transcription factors involved in TGF- β -stimulated collagenase-3 promoter activity, an antisense-Fra-1 expression plasmid was transiently transfected with the collagenase-3 promoter construct. As shown in Fig. 5, co-transfection of antisense-Fra-1 plasmid inhibited both the basal and TGF- β 1-stimulated collagenase-3 promoter activity indicating that the inhibition of Fra-1 expression by antisense-Fra-1 may abolish AP-1 transactivation since Fra-1 forms a complex with c-Jun, JunB or JunD that is necessary for AP-1 transactivation.

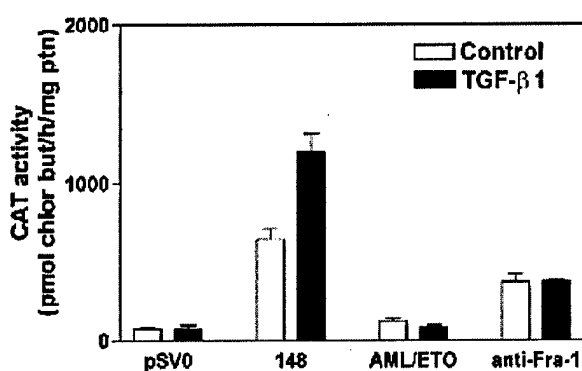
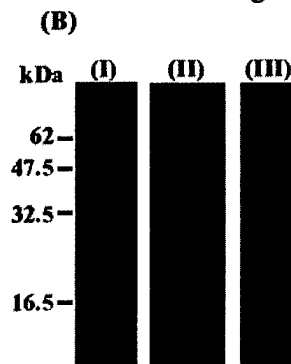
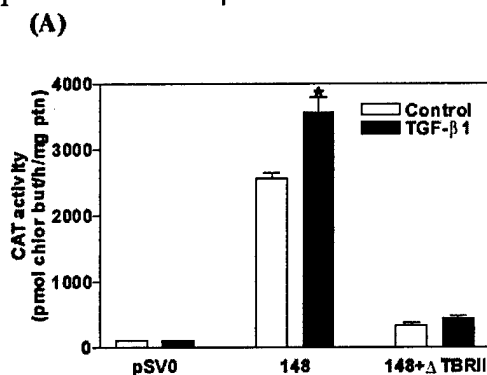


Figure 5. Functional requirement of both the RD and AP-1 sites and their transcription factors for TGF- β 1-stimulated collagenase-3 promoter activity. The wild type collagenase-3 promoter construct (-148) was transiently cotransfected with either pCMV-AML-1/ETO construct or pCMV-anti-Fra-1 construct into MDA-MB231 cells and then treated with control or TGF- β 1-containing media for 24 h, and assayed for CAT activity. The total amount of DNA used for all transfection with or without the expression constructs was equalized with pCMV.

Data represent mean \pm S.E. of three experiments.

TGF- β 1-stimulated collagenase-3 promoter activation depends on TGF- β receptors - Two different transmembrane protein serine/threonine kinases, known as receptor types I and II, are brought together by TGF- β , which acts as a receptor assembly factor. In the ligand-induced complex, receptor II phosphorylates the GS region, resulting in activation of the receptor I kinase. In order to determine the specificity of TGF- β -signaling mediated by TGF- β receptors for collagenase-3 promoter activity, the collagenase-3 promoter construct was transiently co-transfected into MDA-MB231 cells with a dominant-negative TGF- β type II receptor expression plasmid. As shown in Fig. 6A, overexpression of dominant-negative TGF- β type II receptor resulted in significant decreases in basal, as well as TGF- β 1-stimulated collagenase-3 promoter activity. The reduced basal promoter activity by overexpression of dominant negative TGF- β type II receptor in MDA-MB231 cells could possibly be a result of inhibition of endogenous TGF- β signaling. To explore this possibility we used Western blot analysis to detect the expression of TGF- β 1 in MDA-MB231 cells. As shown in Fig. 6B, three major bands of the sizes of 17



kDa, 45 kDa, and 65 kDa corresponding to the biologically active peptide and precursor forms of TGF- β 1 were detected by a specific TGF- β 1 antibody. Thus, the endogenous TGF- β 1 contributes the high basal expression of collagenase-3 in MDA-MB231 cells.

Figure 6. The TGF- β 1-stimulated collagenase-3 promoter activity is mediated by TGF- β receptors. **A,** The wild type collagenase-3 promoter construct (-148) was transiently cotransfected with pCMV-TGF- β type II receptor mutant (TBRII) into MDA-MB231 cells for 24 h, and assayed for CAT activity. The total amount of DNA used for all transfection with or without the expression constructs was equalized with pCMV. Data represent mean \pm S.E. of three experiments. *Significant difference compared with control ($P < 0.001$). **B,** Whole cell extracts were prepared from MDA-MB231 cells and subjected to Western blot analysis as described in methods section. Blots were incubated overnight with either IgG (I) or anti-TGF- β 1 (II) or anti-TGF- β 1 (III) that had been preadsorbed for 30 min with 100X peptide against which it was raised. TGF- β 1 was detected by ECL system.

Activation of ERK1/2 and p38 MAPKs in MDA-MB231 cells by TGF- β 1- Although TGF- β signaling is usually by the Smad pathway in most of the cells studied, some of the biological actions of TGF- β are also mediated by activation of MAPK signaling pathways. To study the role of MAPK (ERK1/2, p38, and JNK) pathways in the regulation of collagenase-3 expression in MDA-MB231 cells, we first determined the activation of these MAPKs by Western blot analysis of cellular proteins at various time points (0, 15, 60, 120, and 240 min) after exposure to TGF- β 1 using antibodies against the active, phosphorylated forms of these MAPKs. TGF- β 1 increased the phosphorylation of ERK1/2 (1.7 fold) at 15 min of stimulation and this reached a maximum at 60 min (2.6 fold). p38 MAPK phosphorylation was increased to a 7.8 fold stimulation at 60 min of TGF- β 1 treatment and declined thereafter. In contrast to the ERK1/2 and p38 MAPKs, there was no phosphorylation of JNK upon TGF- β 1 treatment (see attached paper, Selvamurugan et al., 2002).

Inhibition of TGF- β 1-stimulated collagenase-3 mRNA expression by ERK1/2 and p38 inhibition- To further elucidate the specific roles of MAPKs in mediating the stimulation of collagenase-3 expression by TGF- β 1, we used selective chemical inhibitors of these MAPKs. MDA-MB231 cells were pretreated with PD98059 (25 μ M), a specific inhibitor of ERK1/2 kinases MEK1/2, or SB203580 (25 μ M), a selective inhibitor of p38 MAPK for 30 min and then treated with TGF- β 1 for 24 h. Total RNA was isolated and subjected to real time quantitative PCR. Both ERK1/2 and p38 MAPK inhibitors blocked TGF- β 1-stimulated collagenase-3 mRNA levels from a fold of 2.876 ± 0.617 to a fold of 1.555 ± 0.306 and 1.603 ± 0.085 , respectively. In order to show the specificity of MAPK inhibitors, we used the rat osteoblastic cells (UMR 106-01) treated with PTH (10^{-8} M). Both ERK1/2 and p38 MAPK inhibitors had no effect on PTH-induced collagenase-3 expression in UMR 106-01 cells (see attached paper, Selvamurugan et al., 2002).

There is increased c-Jun, JunB, and Cbfa1/Runx2 proteins in response to TGF- β 1- To determine whether the increased mRNA level in response to TGF- β 1 is correlated with protein expression, cell lysates from MDA-MB231 cells treated with or without TGF- β 1 were carried out for Western blot analysis. The relative levels of c-Jun, JunB, and Cbfa1/Runx2 proteins were quantitated by scanning densitometry and corrected for the levels of α -tubulin. The fold stimulation was calculated over control (0 min). TGF- β 1 maximally stimulated only c-Jun (2-fold), JunB (9-fold), and Cbfa1 (1.5 fold) expression at 2 h (Figs. 7A, B, & C). There was no detectable significant change in the levels of other Fos and Jun family members (data not shown). Since Cbfa1 is a bone specific transcription factor and we have found its RNA and protein expressed in mammary epithelial cells (MDA-MB231), we wanted to confirm the presence of Cbfa1 protein in MDA-MB231 cells. Whole lysates prepared from HeLa, MDA-MB231, and Saos2 cells were subjected to Western blot analysis. HeLa cells (human fibroblast cells) do not express Cbfa1. Blots were incubated with either IgG or anti-Cbfa1 or anti-Cbfa1 that had been preadsorbed with Cbfa1 peptide. Cbfa1 with a mass of 60 kDa was expressed in MDA-MB231 as well as the human osteosarcoma, Saos2 (Fig. 7D).

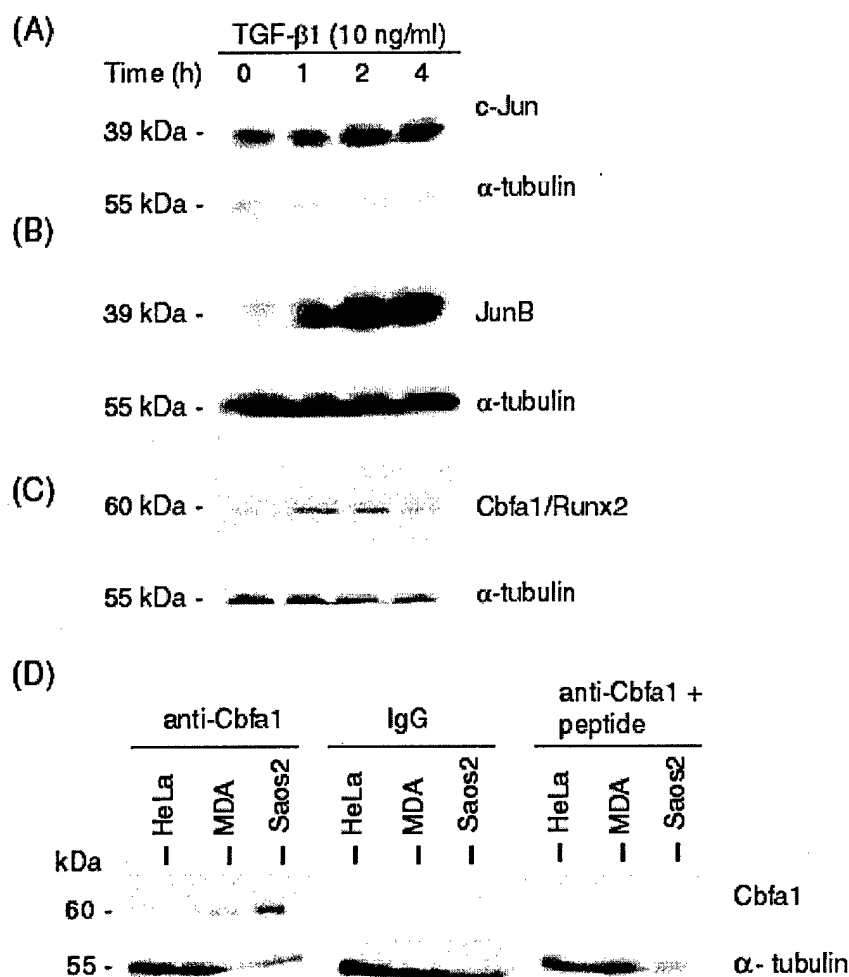


Figure 7. TGF- β 1 stimulates c-Jun, JunB, and Cbfa1/Runx2. (A) MDA-MB231 cells were treated with control or TGF- β 1-containing media for indicated time. Total lysates were prepared and subjected to Western blot analysis using the antibodies as mentioned in the figure. The relative levels of proteins were normalized to α -tubulin and TGF- β 1-fold induction was calculated over control. (B) Whole cell extracts from HeLa, MDA-MB231, and Saos2 cells were subjected to Western blot analysis as described in the methods section. Blots were incubated overnight with either anti-Cbfa1 or IgG or anti-Cbfa1 that had been preadsorbed for 30 min with 100X peptide against which it was raised.

Smad3 physically interacts with both JunB and Cbfa1/Runx2- From the above results, it is evident that there are increased levels of c-Jun, JunB, and Cbfa1 in response to TGF- β 1 in metastatic breast cancer cells. c-Jun and JunB bind to the proximal AP-1/RD site and Cbfa1 binds to the distal RD site. To determine whether there is physical interaction between these proteins, a co-immunoprecipitation assay was used. COS-7 cells were transiently transfected with eukaryotic expression constructs of JunB and c-Myc-tagged Cbfa1. Since TGF- β 1 increased JunB substantially more than c-Jun (Fig. 7B), we used only the JunB expression construct. Total cellular lysates were prepared and incubated with IgG, anti-JunB, or anti-c-Myc. The immunoprecipitates were probed with either anti-JunB or anti-c-Myc antibodies. Fig. 8A shows that JunB and Cbfa1 do not interact with each other.

Recently we have shown that Smad3 is necessary for TGF- β 1-stimulated promoter activity in MDA-MB231 cells. Since JunB and Cbfa1 do not physically interact, we speculated that Smad3 could mediate this interaction. In order to identify this interaction, JunB, c-Myc-Cbfa1, Flag-Smad3, and Flag-Smad4 eukaryotic expression constructs were transiently transfected into COS-7 cells. Total lysates were subjected to immunoprecipitation with IgG, anti-JunB, or anti-c-Myc antibodies and followed by immunoblot with anti-Flag antibody (Fig. 8B). It is evident Smad3, not Smad4 interacts with both JunB and Cbfa1. Similarly, lysates were also immunoprecipitated with either IgG or anti-Flag antibody and immunoblotted with either anti-JunB or anti-c-Myc antibodies (Fig. 8C). This further confirms that Smad3 interacts with both JunB and Cbfa1. In order to verify that Smad4 does not interact with JunB and Cbfa1, lysates prepared from COS-7 cells that had been transfected with JunB, c-Myc-Cbfa1, Flag-Smad3, and Flag-Smad4 were immunoprecipitated with mouse IgG, rabbit IgG, anti-JunB, anti-c-Myc, and anti-Flag antibodies. The blot was probed with anti-Smad4 antibody (Fig. 8D). The results indicate

that Smad4 does not interact with either JunB or Cbfa1. We have also co-transfected c-Myc or Flag tagged vectors as controls and there was no interaction contributed by these tags (data not shown).

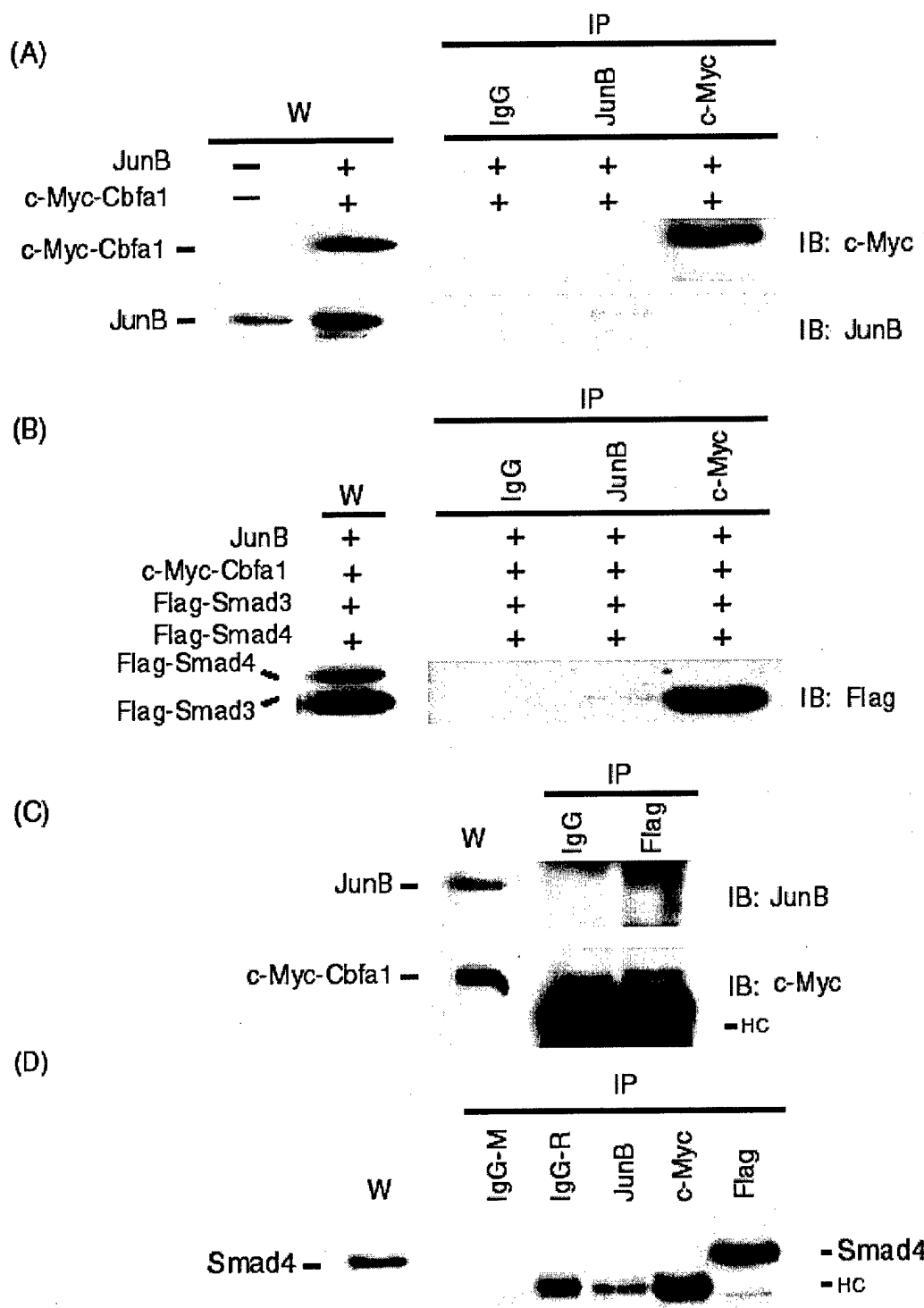


Figure 8. Smad3 interacts with both JunB and Cbfa1/Runx2. (A) Cell lysates from transiently transfected COS-7 cells were subjected to immunoprecipitation with either IgG or anti-JunB or anti-c-Myc antibodies and then immunoblotted using anti-JunB or anti-c-Myc antibody that recognizes Cbfa1/Runx2. (B) Cell lysates from transiently transfected COS-7 cells were subjected to immunoprecipitation with either IgG or anti-JunB or anti-c-Myc antibodies and then immunoblotted using anti-Flag antibody that recognizes Smad3 and Smad4. (C) Cell lysates as mentioned above were

subjected to immunoprecipitation with either IgG or anti-JunB antibody and then immunoblotted using anti-JunB and anti-c-Myc antibodies. (HC-heavy chain) (D) Cell lysates as mentioned above were subjected to immunoprecipitation with either IgGs (mouse or rabbit) or anti-JunB or c-Myc or anti-Flag antibodies and then immunoblotted using anti-Smad4. Expression levels of proteins were determined by immunoblotting aliquots of total cell lysates.

Key Research Accomplishments:

- The construct pCMV-MMP-1-V5 containing the human cytomegalovirus promoter sequence and a 1.65 kb MMP-1 (human matrix metalloproteinase-1 or collagenase-1) cDNA followed by a V5-epitope tag was made.
- Collagenase-3 is efficiently expressed in COS-7 cells under the control of CMV promoter.
- A transgenic construct pOC-MMP-1-V5 containing a ~1 kb rat osteocalcin promoter fragment and a 1.65 kb MMP-1 cDNA fragment followed by a V5-epitope tag was constructed.
- TGF- β 1 (transforming growth factor-- β 1) stimulates collagenase-3 expression in the human breast cancer cell line MDA-MB231.
- TGF- β 1 stimulation of collagenase-3 expression in MDA-MB231 cells requires *de novo* protein synthesis.
- The RD (runt domain binding sequence) and AP-1 (activator protein-1) sites are necessary for TGF- β 1-stimulated collagenase-3 promoter activity in human breast cancer cells.
- Dominant negative inhibitors identify the functional requirement of both the RD and AP-1 sites for TGF- β 1-stimulated collagenase-3 promoter activity.
- TGF- β 1-stimulated collagenase-3 promoter activation depends on TGF- β receptors.
- TGF- β 1-stimulated collagenase-3 expression requires both the Smad and the MAPK pathways.
- There is increased c-Jun, JunB, and Cbfa1/Runx2 proteins in response to TGF- β 1.
- Smad3 physically interacts with both JunB and Cbfa1.

Reportable Outcomes:

Manuscripts:

Transcriptional activation of collagenase-3 by transforming growth factor-beta1 is via MAPK and Smad pathways in human breast cancer cells.

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Abstracts:

Transforming Growth Factor- β 1 stimulation of collagenase-3 expression in human breast cancer cells.

N. Selvamurugan, Z. Fung, and N. C. Partridge

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To be presented at the 25th Annual meeting of American Association for Bone and Mineral Research, September 19-23, 2003, Minneapolis, Minnesota, USA.

Conclusions:

1. The application of a transgenic mouse model will contribute greatly to the understanding of the pathogenesis of bone metastasis. Identification of the exact nature of these tumor-bone interactions may not only generate valuable information on underlying regulatory mechanisms in invasion and bone metastasis but can also be of value in the development of therapeutic strategies.

2. An intensive drug discovery program led to many clinical trials of MMP inhibitors for cancer therapy. However, these trials have largely been disappointing. A greater understanding of the regulatory mechanisms that control MMP transcription, activation and inhibition will provide several new avenues for therapeutic intervention.

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Appendix:

1. Transcriptional activation of collagenase-3 by transforming growth factor-beta1 is via MAPK and Smad pathways in human breast cancer cells.

N. Selvamurugan, Z. Fung, and N. C. Partridge (2002)

FEBS Lett. 532, 31-35.

Transcriptional activation of collagenase-3 by transforming growth factor- β 1 is via MAPK and Smad pathways in human breast cancer cells

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Abstract Transforming growth factor (TGF)- β 1, a crucial molecule in metastatic bone cancer, stimulates collagenase-3 expression in the human breast cancer cell line, MDA-MB231. Cycloheximide inhibited this stimulation, indicating that de novo protein synthesis was essential for this response. We examined whether mitogen-activated protein kinase (MAPK) and/or Smad pathways are involved in TGF- β 1-stimulated collagenase-3 expression in MDA-MB231 cells. Biochemical blockade of extracellular regulated kinase-1/2 and p38 MAPK pathways partially abolished TGF- β 1-stimulated collagenase-3 mRNA expression; whereas overexpression of a dominant negative form of Smad3 completely blocked the TGF- β 1-response. These data indicate that TGF- β 1-induced MAPK and Smad pathways are involved in TGF- β 1-stimulated collagenase-3 expression in MDA-MB231 cells.

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Key words: Extracellular matrix; Collagenase-3; Transforming growth factor- β 1 signaling; Breast cancer metastasis

1. Introduction

Matrix metalloproteinases (MMPs) are a family of secreted or transmembrane proteinases that have been implicated in multiple physiological and pathological processes related to extracellular matrix (ECM) turnover, such as normal growth and development, wound healing, angiogenesis, and joint destruction in arthritis [1–4]. MMPs also play an integral role in tumor invasion that is characterized by increased motility of epithelial cells and growth of metastasized tumor cells [5]. Recent studies have shown that collagenase-3 (MMP-13) is overexpressed in a variety of malignant tumors. Human collagenase-3 was originally identified in human breast carcinoma cells [6] and is homologous to rat collagenase-3 [7]. Due to the wide substrate specificity of collagenase-3, the expression of collagenase-3 is very restricted and appears to be subjected

to stringent regulatory mechanisms. During tumor growth these controls are lost and tumor cells acquire the ability to produce this protease under stimulation by different factors, including cytokines, growth factors, and tumor promoters. Among them, interleukin (IL) 1 α and IL-1 β are potential candidates for inducing the expression of this MMP gene in breast carcinomas [8,9].

Transforming growth factor (TGF)- β , a multipotent cytokine enriched in bone matrix, has a wide range of physiological and pathological effects [10–13]. TGF- β 1, a crucial molecule in bone metastatic cancer, appears to help guide completion of the bone remodeling cycle. It could also promote breast cancer metastasis by acting directly on the tumor cells [14] via production of parathyroid hormone (PTH)-related protein (PTHrP) that is the critical mediator of bone destruction. The molecular mechanisms responsible for osteolytic metastases are complex and involve bi-directional interactions between tumor cells and bone. One of the possible ways that TGF- β 1 is involved in bone destruction is via regulation of MMP activity in metastasizing bone cancer cells. In the present study we show TGF- β 1 stimulation of collagenase-3 mRNA expression and secretion in the human breast cancer cell line, MDA-MB231, and the signaling pathways mediating this response.

2. Materials and methods

2.1. Cell culture and growth factor stimulation

MDA-MB231 cells were obtained from ATCC (American Type Culture Collection). The cells were maintained in culture in Dulbecco's modified essential medium (DMEM-F12) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were treated with TGF- β 1 (Invitrogen) or control vehicle in the absence of serum. Inhibitors used in these experiments included the MEK-1 inhibitor PD98059 (Calbiochem), and the p38 mitogen-activated protein kinase (MAPK) inhibitor, SB203580 (Calbiochem). Pretreatments with these inhibitors were for 30 min.

2.2. Semiquantitative RT-PCR

Total RNA was prepared using the Qiagen RNeasy kit. RNA obtained from control and TGF- β 1-treated MDA-MB231 cells was used for reverse transcription polymerase chain reaction (RT-PCR) by SuperScript One Step RT-PCR kit (Invitrogen). The oligonucleotides (sense 5'-CCTCCTGGGCCAAATTATGGAG-3' and antisense 5'-CAGCTCCGCATCAACCTGCTG-3') corresponding to human collagenase-3 were used for specific amplification of a 392 bp fragment of collagenase-3 mRNA. The initial temperature for RT-PCR was 50°C (30 min) and 94°C (2 min) and then 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s), and elongation (68°C, 1 min). The final extension was at 68°C (7 min). The products were analyzed on a 2% agarose gel.

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Abbreviations: MMP, matrix metalloproteinase; TGF- β 1, transforming growth factor- β 1; ECM, extracellular matrix; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; RT-PCR, reverse transcription polymerase chain reaction; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase

2.3. Northern blot analysis

Twenty μg of total RNA per lane was electrophoresed on a 1% agarose, 2.2 M formaldehyde gel in MOPS buffer (40 mM MOPS, pH 7.0, 10 mM sodium acetate and 1 mM EDTA). RNA was transferred to Zeta-Probe GT-membrane (Bio-Rad) and hybridized in 50% formamide, $5\times\text{SSC}$, $10\times\text{Denhardt's}$, 0.1% SDS, 0.05 M NaPO_4 and 100 $\mu\text{g/ml}$ salmon sperm DNA at 42°C . cDNA probes used for hybridization were labeled either with random priming by Prime-a-Gene kit (Promega) or by nick translation kit (Promega). Northern blots were visualized by exposure to film and quantitated by exposure to phosphor screens and analysis in a phosphorimager.

2.4. Quantitative RT-PCR

Total RNA was prepared using the Qiagen RNeasy kit. Reverse transcriptase reaction was carried out using the TaqMan Reverse Transcription reagents (Roche). PCR reactions were performed according to the real-time PCR machine manufacturer's instructions (DNA Engine Opticon, MJ Research, MA, USA), which allow real-time quantitative detection of the PCR product by measuring the increase in SYBR green fluorescence caused by binding of SYBR green to double-stranded DNA. The SYBR green kit for PCR reactions was purchased from Perkin Elmer Applied Biosystems. Primers for human collagenase-3 and β -actin were designed using the Primer-Express software (Perkin Elmer Applied Biosystems).

2.5. Western blot analysis

Whole cell lysates from MDA-MB231 cells containing 50 μg of total protein in lysis buffer were electrophoresed by 12% SDS-PAGE. The proteins were transferred electrophoretically to polyvinylidene difluoride membrane (Bio-Rad). After blocking in Tween-Tris-buffered saline (0.1% Tween 20, 138 mM NaCl, 5 mM KCl, and 25 mM Tris-HCl, pH 8.0) containing 5% (w/v) non-fat dry milk, the membrane was exposed to primary antibody overnight at 4°C . The

membrane was washed and exposed to horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (diluted 1:5000). The antigen-antibody complexes were detected by enhanced chemiluminescence (Amersham Biosciences).

2.6. Transient transfection and chloramphenicol acetyltransferase (CAT) activity

The plasmid DNAs were transiently transfected into breast cancer cells using Lipofectamine (Invitrogen). Briefly, cells were plated at 4×10^5 cells/well in six-well plates in DMEM-F12 containing 10% FBS. The following day, the cells were transfected with 1 μg DNA and 5 μl Lipofectamine per plate in 1 ml of serum-free DMEM-F12. After 16 h, 1 ml of DMEM-F12 containing 10% FBS was added. After 24 h, the cells were treated with either control or TGF- β -containing medium for 24 h. CAT activity was measured by reacting 50 μl of cell lysate in duplicate in a 100 μl reaction volume consisting of final concentrations of 250 μM *n*-butyryl-coenzyme A and 23 mM [^{14}C]chloramphenicol (0.125 $\mu\text{Ci/assay}$). Butylated chloramphenicol was removed by pre-extraction with 200 μl of mixed xylenes. Butylated chloramphenicol retained in the final organic layer was determined by scintillation counting. The values were normalized to protein as determined by the Bradford dye binding (Bio-Rad) method. A standard curve using purified CAT was performed every experiment to determine the linear range of the enzyme assay.

3. Results

3.1. TGF- β 1 stimulates collagenase-3 mRNA expression in MDA-MB231 cells and it requires de novo protein synthesis

To study the effect of TGF- β 1 on expression of collagenase-

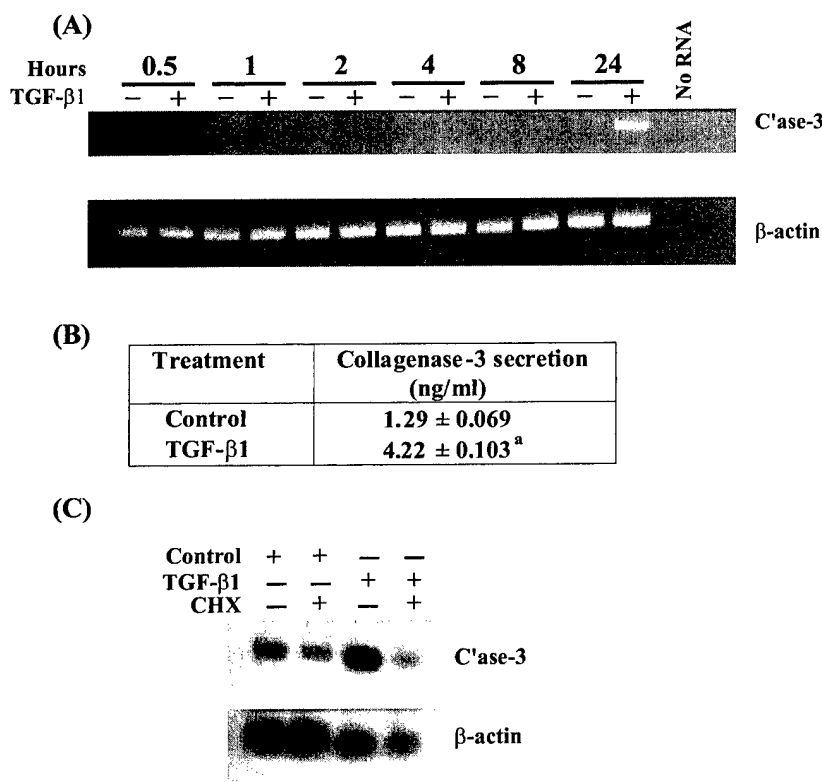


Fig. 1. TGF- β 1 stimulates collagenase-3 expression in MDA-MB231 cells and it requires de novo protein synthesis. A: MDA-MB231 cells were serum starved for 24 h and then treated with TGF- β 1 (10 ng/ml) at different time periods as indicated. Sense and antisense oligos for human collagenase-3 (C'ase-3) and β -actin were used for RT-PCR. B: MDA-MB231 cells were serum starved for 24 h and then treated with control medium or medium containing TGF- β 1 (10 ng/ml) for 24 h. The collagenase-3 levels in the media were measured using an ELISA kit (Amersham Pharmacia). Data represent mean \pm S.E.M. of three replicate plates. The statistical analysis was performed using Student's *t*-test and Prism 3.0. ^aSignificant difference compared with control ($P < 0.001$). C: MDA-MB231 cells were serum starved for 24 h and then treated with control medium or medium containing TGF- β 1 (10 ng/ml) for 24 h in the presence or absence of cycloheximide (30 $\mu\text{g/ml}$, added 1 h before TGF- β 1 treatment). Total RNA was subjected to Northern blot analysis and probed with labeled human collagenase-3 or β -actin cDNAs.

3 in human breast cancer, MDA-MB231 cells were treated with TGF- β 1 either at different concentrations for 24 h or for different time periods with 10 ng/ml. Total cellular RNAs were purified and analyzed by semi-quantitative RT-PCR. TGF- β 1 maximally stimulated collagenase-3 RNA expression at 10 ng/ml concentration in MDA-MB231 cells (data not shown) and requires 24 h for this effect (Fig. 1A). The identity of collagenase-3 (392 bp) and β -actin (661 bp) PCR products was confirmed by transferring PCR products to filters and hybridizing to labeled human collagenase-3 and β -actin cDNA probes, respectively.

An ELISA was then performed using an antibody to human collagenase-3 confirming increased secretion of collagenase-3 into the medium from TGF- β 1-treated MDA-MB231 cells (Fig. 1B). To determine if the TGF- β 1-mediated increase in collagenase-3 mRNA is a primary response, we used the protein synthesis inhibitor cycloheximide. As shown in Fig. 1C, cycloheximide inhibited TGF- β 1 stimulation of collagenase-3 mRNA, indicating that de novo protein synthesis is required for this response.

3.2. Activation of ERK1/2 and p38 MAPKs in MDA-MB231 cells by TGF- β 1

Although TGF- β signaling is usually by the Smad pathway

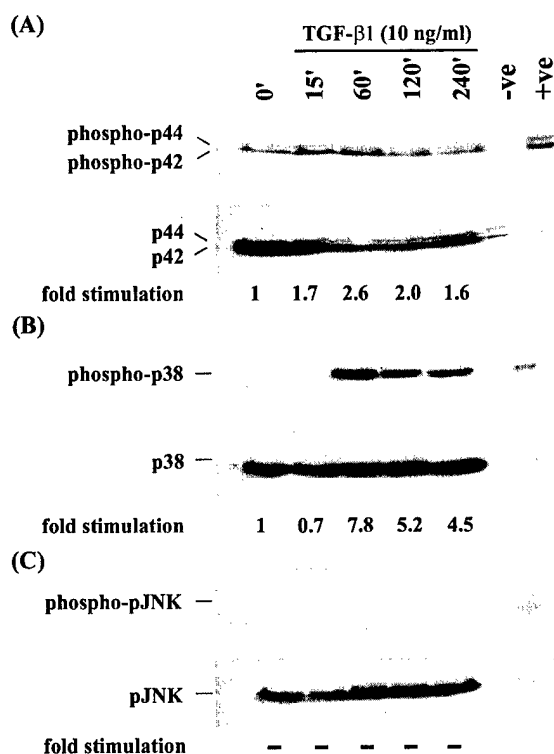


Fig. 2. MAPK pathway activation by TGF- β 1. MDA-MB231 cells were incubated with TGF- β 1 (10 ng/ml) for the times indicated. A: Phosphorylation of ERK1/2 from whole cell lysates was determined using Western blot with anti-phospho and anti-total ERK1/2 antibodies. B: Phosphorylation of p38 MAPK from whole cell lysates was determined using Western blot with anti-phospho and anti-total p38 antibodies. C: Phosphorylation of JNK MAPK from whole cell lysates was determined using Western blot with anti-phospho and anti-total MAPK antibodies. Cell lysates from C6 cells stimulated with or without anisomycin served as positive (+) and negative (-) controls, respectively. The levels of phospho MAPKs and total MAPKs were quantitated by scanning densitometry and the fold stimulation was calculated over control (0 min).

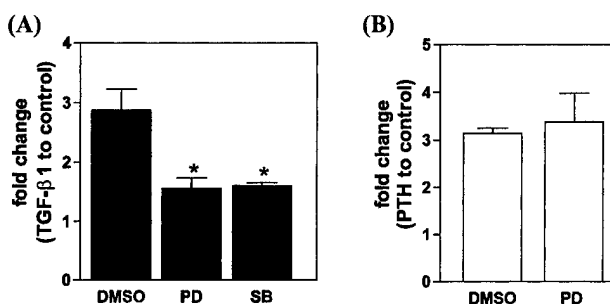


Fig. 3. Inhibition of TGF- β 1-stimulated collagenase-3 mRNA expression by ERK1/2 and p38 MAPK inhibition. A: MDA-MB231 cells were pretreated with dimethylsulfoxide (DMSO) or PD98059 or SB203580 for 30 min and incubated in the presence or absence of TGF- β 1 (10 ng/ml) for 24 h. Total RNA was isolated and subjected to real-time quantitative RT-PCR using human collagenase-3 and β -actin primers. The relative levels of mRNAs were normalized to β -actin and the change in the levels of collagenase-3 mRNA expression in response to TGF- β 1 was calculated as fold change over control. The data are represented as mean \pm S.D. ($n=3$) and were analyzed by ANOVA using Prism 3.0. *Significant difference compared with control (DMSO) ($P<0.05$). B: UMR 106-01 cells were pretreated with DMSO or PD98059 or SB203580 for 30 min and incubated in the presence or absence of PTH (10^{-8} M) for 24 h. Total RNA was isolated and subjected to real-time quantitative RT-PCR using human collagenase-3 and β -actin primers as mentioned above.

in most of the cells studied, some of the biological actions of TGF- β are also mediated by activation of MAPK signaling pathways. To study the role of MAPK (extracellular signal-regulated kinase (ERK) 1/2, p38, and JNK) pathways in the regulation of collagenase-3 expression in MDA-MB231 cells, we first determined the activation of these MAPKs by Western blot analysis of cellular proteins at various time points (0, 15, 60, 120, and 240 min) after exposure to TGF- β 1 using antibodies against the active, phosphorylated forms of these MAPKs (Cell Signaling Technology). The levels of activated MAPKs (p-ERK1/2, p-p38, and p-JNK) were quantitated by scanning densitometry and corrected for the levels of total MAPKs (ERK1/2, p38, and JNK) in the same samples. As shown in Fig. 2A, TGF- β 1 increased the phosphorylation of ERK1/2 (1.7-fold) at 15 min of stimulation and this reached a maximum at 60 min (2.6-fold). p38 MAPK phosphorylation was increased to a 7.8-fold stimulation at 60 min of TGF- β 1 treatment and declined thereafter (Fig. 2B). In contrast to the ERK1/2 and p38 MAPKs, there was no phosphorylation of JNK upon TGF- β 1 treatment (Fig. 2C).

3.3. Inhibition of TGF- β 1-stimulated collagenase-3 mRNA expression by ERK1/2 and p38 inhibition

To further elucidate the specific roles of MAPKs in mediating the stimulation of collagenase-3 expression by TGF- β 1, we used selective chemical inhibitors of these MAPKs. MDA-MB231 cells were pretreated with PD98059 (25 μ M), a specific inhibitor of ERK1/2 kinases MEK1/2, or SB203580 (25 μ M), a selective inhibitor of p38 MAPK, for 30 min and then treated with TGF- β 1 for 24 h. Total RNA was isolated and subjected to real-time quantitative PCR. Both ERK1/2 and p38 MAPK inhibitors blocked TGF- β 1-stimulated collagenase-3 mRNA levels from a fold of 2.876 ± 0.617 to a fold of 1.555 ± 0.306 and 1.603 ± 0.085 , respectively (Fig. 3A). In order to show the specificity of MAPK inhibitors, we used rat osteoblastic cells (UMR 106-01) treated with PTH (10^{-8} M).

Both ERK1/2 (Fig. 3B) and p38 MAPK (data not shown) inhibitors had no effect on PTH-induced collagenase-3 expression in UMR 106-01 cells.

3.4. TGF- β 1-stimulated collagenase-3 promoter activity depends on the Smad pathway

To determine the functional role of the Smad pathway for TGF- β 1-stimulated collagenase-3 expression in MDA-MB231 cells, the rat collagenase-3 promoter containing CAT as a reporter gene [15] was transiently transfected into MDA-MB231 cells along with a dominant-negative form of Smad3 (Smad3M) expression plasmid. The rat collagenase-3 promoter is similar to the human collagenase-3 promoter and most of the regulatory elements present in the rat and human collagenase-3 promoters are highly conserved [15,16]. Overexpression of the Smad mutant significantly abolished both the basal and TGF- β 1-stimulated collagenase-3 promoter activity indicating that mutant Smad3 competes with endogenous Smad3 to form a heterodimer with Smad2 and Smad4, thus blocking translocation of the complex into the nucleus (Fig. 4A). In order to determine the specificity of TGF- β

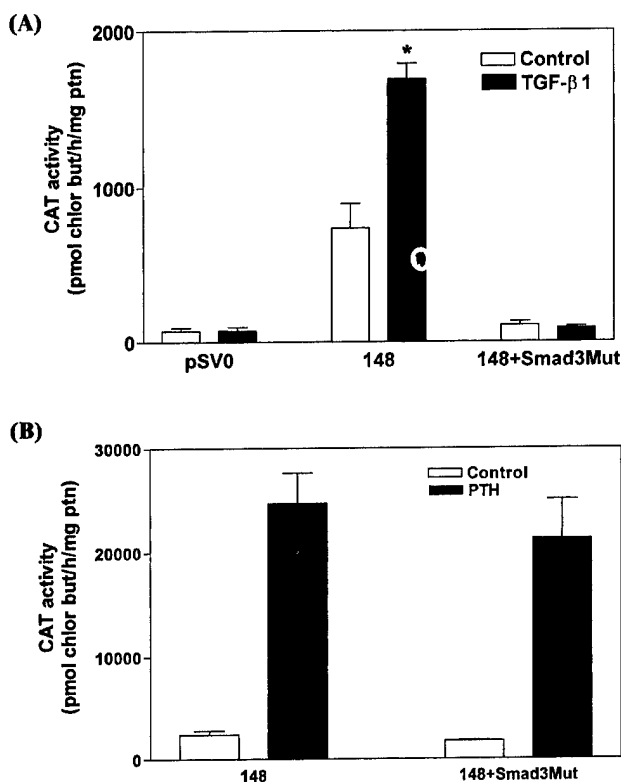


Fig. 4. TGF- β 1-stimulated collagenase-3 promoter activation depends on the Smad pathway. A: The wild type collagenase-3 promoter construct (-148) was transiently cotransfected with pCMV-Smad3Mutant (Smad3Mut) construct into MDA-MB231 cells and then treated with control or TGF- β -containing medium for 24 h, and assayed for CAT activity. The total amount of DNA used for all transfections with or without the expression constructs was equalized with pCMV. Data represent mean \pm S.D. of three experiments. The data were analyzed by Student's *t*-test and Prism 3.0. *Significant difference compared with control ($P < 0.001$). B: The wild type collagenase-3 promoter construct was transiently cotransfected with pCMV-Smad3Mutant construct into UMR 106-01 cells and then treated with control or PTH (10^{-8} M)-containing medium for 24 h, and assayed for CAT activity. Data represent mean \pm S.D. of three experiments.

signaling that is mediated through the Smad pathway for collagenase-3 promoter activity in MDA-MB231 cells, we used the rat osteoblastic cells, UMR 106-01, with PTH (10^{-8} M) treatment. As shown in Fig. 4B, cotransfection of Smad3M did not inhibit PTH-induced collagenase-3 promoter activity in UMR 106-01 cells indicating that Smad-mediated TGF- β signaling in MDA-MB231 cells is specific.

4. Discussion

IL-1 α and IL-1 β are potential candidates for inducing the expression of collagenase-3 in breast carcinomas [8,9]. TGF- β , a multipotent growth factor highly enriched in bone matrix, induces collagenase-3 expression in human fibroblasts [9]. We here demonstrated that treatment of human breast cancer cells with TGF- β also stimulates collagenase-3 mRNA expression and secretion. Collagenase-3-driven ECM proteolysis may support cancer cell growth both biochemically, by exposing mitogenic factors, and physically, by providing space for the proliferating cells. Since TGF- β -stimulated collagenase-3 expression requires de novo protein synthesis and the collagenase-3 promoter contains an AP-1 site [15], AP-1 transactivation may be required for collagenase-3 expression. AP-1 complex composition can selectively regulate gene transcription and differential expression of Fos and Jun family members could play a role to regulate the expression of downstream target genes [9,17]. We previously showed that differential temporal stimulation of the AP-1 family members may be responsible for collagenase-3 expression in osteoblastic and non-osteoblastic cells [18].

Smad proteins have been shown to mediate the transcriptional activation of various TGF- β -responsive genes such as collagen [19], the tissue plasminogen activator inhibitor [20], the JunB proto-oncogene [21], and the p21/WAF1/Cip1 cell cycle inhibitor [22]. Smad proteins are the main cytoplasmic signaling pathways in TGF- β 1-stimulated collagenase-3 expression in osteoarthritic chondrocytes [23]. The Smad proteins are central elements in the TGF- β -receptor signaling pathway but are not the sole pathway activated by this receptor complex. TGF- β family members often require the presence of parallel or synergistic pathways to the Smads to carry out their full biological effects, and diversity of the Smad-interacting partners may contribute to signal specificity [24]. In gingival fibroblasts, TGF- β 1 activates both ERK1/2 and p38 MAPK pathways but collagenase-3 expression depends only on the activity of p38 MAPK and the presence of functional AP-1 dimers [25]. The enhancement of collagenase-3 expression by TGF- β 1 is also mediated by the p38 MAPK pathway in transformed keratinocytes [26]. In the present study, we evaluated the effect of inhibiting the ERK1/2 or the p38 MAPK pathways on TGF- β -stimulated collagenase-3 mRNA expression in MDA-MB231 cells. Either of them did not completely block the TGF- β -stimulated collagenase-3 mRNA expression indicating the participation of another pathway (Smad) for this effect. Overexpression of the dominant-negative form of Smad3 completely blocked the TGF- β stimulation of the collagenase-3 promoter suggesting that the Smad pathway represents a major pathway for TGF- β in these cells.

The interactions between MAPK and Smad pathways downstream of the TGF- β receptor may be complex. Smad2 and Smad3 are direct substrates for phosphorylation by active

TGF- β type I receptor. Because a dominant-negative form of Smad3 fully suppressed TGF- β responsiveness, activation of the ERK and p38 MAPK pathways would result from tyrosine kinase receptors rather than from serine/threonine kinase receptors (TGF- β receptor). Recently it has been shown that TGF- β -stimulated fibronectin expression is mediated by epidermal growth factor receptor transactivation, and subsequent activation of ERK and p38 MAPKs [27]. There may be cross-talk between the MAPK and Smad pathways for TGF- β -stimulated collagenase-3 expression in MDA-MB231 cells. For example, MAPKs phosphorylate Smad2/3 proteins other than the SSXS motif activated by the TGF- β type I receptor. Cross-talk between Smad, ERK1/2, and p38 MAPK pathways for TGF- β induction of the aggrecan gene has been reported [28]. TGF- β 1 stimulation of PTHrP is also dependent on Smad and MAPK pathways [29]. Overall, our studies demonstrate transcriptional activation of the collagenase-3 gene by TGF- β 1 in human breast cancer cells and that transcriptional activation is mediated by both the MAPK and Smad pathways.

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